

WHAT IS CLAIMED IS:

1. A method for isolating a modified peptide from a complex mixture of peptides, said method comprising the steps of:
 - 5 (a) obtaining a proteinaceous preparation from an organism, wherein said proteinaceous preparation comprises modified peptides from two or more different proteins;
 - (b) contacting said proteinaceous preparation with at least one immobilized modification-specific antibody; and
 - 10 (c) isolating at least one modified peptide specifically bound by said immobilized modification-specific antibody in step (b).
2. The method of claim 1, further comprising the step of (d) characterizing said modified peptide isolated in step (c) by mass
15 spectrometry (MS), tandem mass spectrometry (MS-MS), and/or MS³ analysis.
3. The method of claim 2, wherein said mass spectrometry comprises MALDI-TOF MS, wherein said tandem mass spectrometry comprises LC-
20 MS/MS, and wherein said MS³ analysis comprises LC-MS³.
4. The method of claims 2 or 3, further comprising the step of (e) utilizing a search program to substantially match the spectra obtained for said modified peptide during the characterization of step (d) with the spectra for a
25 known peptide sequence, thereby identifying the parent protein(s) of said modified peptide.

5. The method of claim 1, wherein said proteinaceous preparation comprises a digested biological sample selected from the group consisting of a digested crude cell extract, a digested tissue sample, a digested serum sample, a digested urine sample, a digested synovial fluid sample, and a
5 digested spinal fluid sample.
6. The method of claim 5, wherein said digested preparation is obtained using at least one proteolytic enzyme or chemical cleavage.
- 10 7. The method of claim 6, wherein said proteolytic enzyme is immobilized.
8. The method of claim 6, wherein said proteolytic enzyme is soluble, and wherein said digested preparation is treated with a proteolysis inhibitor
15 prior to said contacting step (b).
9. The method of claim 1, wherein step (a) further comprises pre-purifying said proteinaceous preparation by immobilized metal affinity chromatography (IMAC).
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10. The method of claim 1, wherein said immobilized antibody of step (b) is covalently-linked to a chromatography resin or noncovalently-linked to protein-A- or protein-G-agarose.
- 25 11. The method of claim 10, wherein said resin is contained within a column or micropipette tip.

12. The method of claim 2, wherein said immobilized antibody of step (b) is immobilized in chromatography resin within a column, said column being coupled to a mass spectrometer for said characterization of step (d).

5 13. The method of claim 1, wherein said modification comprises phosphorylation.

14. The method of claim 1, wherein said modified peptide(s) comprise(s) a phosphopeptide.

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15. The method of claim 1, wherein said modification-specific antibody comprises a motif-specific, context-independent antibody that recognizes a motif comprising at least one phosphorylated amino acid.

15 16. The method of claim 15, wherein said motif consists of a single phosphorylated amino acid.

17. The method of claim 15, wherein said motif comprises all or part of a kinase consensus substrate motif or a protein-protein binding motif.

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18. The method of claim 17, wherein said kinase consensus substrate motif is selected from the group consisting of MAPK consensus substrate motifs, CDK consensus substrate motifs, PKA consensus substrate motifs, AKT consensus substrate motifs, PKC consensus substrate motifs, phosphothreonine-X-arginine, and ATM consensus substrate motifs, and wherein said protein-protein binding is a 14-3-3 binding motif or a PDK1 docking motif.

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19. The method of claim 1, wherein said modification-specific antibody is a monoclonal antibody or a polyclonal antibody.

20. The method of claim 1, wherein said modified peptide isolated in step
5 (c) corresponds to a known marker of disease.

21. The method of claim 4, wherein said modified peptide characterized in step (d) comprises an unknown modification site of said parent protein.

10 22. The method of claims 2 or 3, further comprising the step of (e) comparing the modification state of said modified peptide characterized in step (d) with the modification state of a corresponding peptide in a reference sample, thereby to compare protein activation in said proteinaceous preparation with protein activation in said reference sample.

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23. The method of claim 22, wherein said proteinaceous preparation corresponds to a diseased organism and said reference sample corresponds to a normal organism, whereby comparison of protein activation provides information on activation changes resulting from said disease.

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24. The method of claim 22, wherein said proteinaceous preparation is obtained from a tissue biopsy cell or a clinical fluid sample and said reference sample corresponds to a diseased organism, whereby the comparison of protein activation provides information useful for diagnosis of
25 said disease.

25. The method of claim 22, wherein said protein preparation corresponds with an organism or preparation treated with at least one test compound and

said reference sample corresponds with an untreated organism or preparation, whereby the comparison of protein activation provides information on activation changes resulting from treatment with said test compound.

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26. The method of claim 23, wherein the comparison of protein activation identifies the modified peptide characterized in step (d) as corresponding to a parent protein not previously reported as so modified in said disease.

10 27. The method of claim 24 or 25, wherein said disease is cancer.

28. The method of claim 25, wherein said test compound comprises a cancer therapeutic.

15 29. The method of claim 25, wherein said test compound comprises a kinase inhibitor.

30. A method for isolating a phosphopeptide from a complex mixture of peptides, said method comprising the steps of:

- 20 (a) obtaining a proteinaceous preparation from an organism, wherein said proteinaceous preparation comprises phosphopeptides from two or more different proteins;
- (b) fractionating phosphopeptides in said proteinaceous preparation by reversed-phased chromatography to produce a
- 25 fractionated proteinaceous preparation;

- (c) contacting said fractionated proteinaceous preparation with at least one immobilized motif-specific, context-independent antibody that binds a motif comprising at least one phosphorylated amino acid;
- (d) isolating at least one phosphopeptide specifically bound by said
5 immobilized antibody in step (c); and
- (e) characterizing said phosphopeptide isolated in step (d) by mass spectrometry (MS), tandem mass spectrometry (MS-MS), and/or MS³ analysis.
- 10 31. The method of claim 30, further comprising the step of (f) utilizing a search program to substantially match the mass spectra obtained for said phosphopeptide during the characterization of step (e) with the mass spectra for a peptide of one or more known protein(s), thereby identifying the parent protein(s) of said modified peptide.
- 15 32. The method of claim 32, wherein said mass spectrometry comprises MALDI-TOF MS, wherein said tandem mass spectrometry comprises LC-MS/MS, and wherein said MS³ analysis comprises LC-MS³.
- 20 33. The method of claim 32, wherein step (a) further comprises digesting said proteinaceous preparation to produce a complex mixture of peptides.
34. The method of claim 30, wherein said motif-specific, context-independent antibody of step (c) comprises a general phosphotyrosine-
25 specific antibody, a general phosphothreonine-specific antibody, or a general phosphoserine-specific antibody.

35. The method of claim 30, wherein said motif-specific, context-independent antibody of step (c) is specific for a phosphorylated kinase consensus substrate motif or protein-protein binding motif.

5 36. The method of claim 35, wherein said kinase consensus substrate motif is selected from the group consisting of MAPK consensus substrate motifs, CDK consensus substrate motifs, PKA consensus substrate motifs, AKT consensus substrate motifs, PKC consensus substrate motifs, phosphothreonine-X-arginine, ATM/ATR consensus substrate motifs, p85
10 PI3K binding motif, phosphothreonine-proline motif, Arg-X-Tyr/Phe-X-phosphoserine motif, phosphoserine/phosphothreonine-Phe motif, PLK consensus substrate motifs, and DNA damage-induced substrate motifs, and wherein said protein-protein binding is a 14-3-3 binding motif or a PDK1 docking motif.

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37. The method of claim 30, wherein said reversed-phased chromatography of step (b) comprises a C18 column.

38. The method of claim 30, further comprising the step of (f) quantifying
20 said isolated phosphopeptides of step (e).

39. The method of claim 38, wherein step (f) comprises quantifying said isolated phosphopeptides using stable isotope labeling by amino acids in cell culture (SILAC) and/or absolute quantification of peptides (AQUA)
25 techniques.

40. An immunoaffinity isolation device for the isolation of modified peptides a complex mixture, said device comprising a support comprising at

least one modification-specific antibody immobilized to a rigid, non-porous or macroporous resin.

41. The device of claim 40, wherein said support is selected from the group consisting of a thin capillary column having an internal diameter of about 50 to 300 micrometers and a micropipette tip.
42. The device of claim 41, wherein said modification-specific antibody comprises a motif-specific, context-independent antibody.
43. The device of claim 41, wherein said column is adapted to be coupled to an electrospray source on a mass spectrometer.
44. An antibody that binds ubiquitin fusion degradation protein 1 (UFD1) only when phosphorylated at serine 335, but does not substantially bind to UFD1 when not phosphorylated at this residue.
45. An antibody that binds protein-tyrosine phosphatase 1c (PTN6) only when phosphorylated at serine 588, but does not substantially bind to PTN6 when not phosphorylated at this residue.
46. An antibody that binds a protein phosphorylation site listed in Column 5 of Table 5 only when phosphorylated at the phosphorylatable residue indicated in Column 5, but does not substantially bind to the phosphorylation site when not phosphorylated at the indicated residue.
47. An antibody that binds a protein phosphorylation site listed in Column 5 of Table 6 only when not phosphorylated at the phosphorylatable residue

indicated in Column 5, but does not substantially bind to the phosphorylation site when phosphorylated at the indicated residue.

48. An antibody that binds a protein phosphorylation site listed in Column
5 4 of Table 7 only when not phosphorylated at the phosphorylatable residue indicated in Column 4, but does not substantially bind to the phosphorylation site when phosphorylated at the indicated residue.